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(21) International Application Number: PCT/EP97/01117 (22) International Filing Date: 7 March 1997 (07.03.97) (30) Priority Data: 9605274.1 13 March 1996 (13.03.96) GB (71) Applicant (for all designated States except US): DANISCO A/S [DK/DK]; Langebrogade 1, P.O. Box 17, DK-1001 Copenhagen K (DK). (72) Inventors; and (75) Inventors/Applicants (for US only): DAL DEGAN, Florence [FR/DK]; (DK). POULSEN, Peter [DK/DK]; (DK). MARCUSSEN, Jan [DK/DK]; (DK). OXENBAL, Susanne [DK/DK]; Danisco a/s, Langebrogade 1, P.O. Box 17, DK- 1001 Copenhagen K (DK). (74) Agents: HARDING, Charles, Thomas et al.; D. Young & Co., 21 New Fetter Lane, London EC4A 1DA (GB).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i> (88) Date of publication of the international search report: 30 October 1997 (30.10.97)
(54) Title: DNA ENCODING ALPHA-1(1,4)-GLUCAN ACETYL-TRANSFERASE (57) Abstract An enzyme is described. The enzyme has $\alpha(1,4)$ glucan acetyl-transferase activity.		

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A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N9/10 C12N15/11 C12N15/54 C12N15/82 C12P19/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 44 25 688 A (A U F ANALYTIK UMWELTECHNIK F) 18 January 1996 see the whole document	14
X	--- BRAND B. AND BOOS W.: "Maltose transacetylase of Escherichia coli" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 21, 25 July 1991, pages 14113-14118, XP000676505 cited in the application see the whole document	1-15
P,X	--- ROBERTS D. ET AL.: "Sequence of minutes 4-25 of E. coli, AC U82664" EMBL DATABASE, 19 January 1997, HEIDELBERG, XP002036098 see the whole document -----	1-12,15

☐ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p>(54) Title: AN ENZYME</p> <p>(57) Abstract</p> <p>An enzyme is described. The enzyme has $\alpha(1,4)$ glucan acetyl-transferase activity.</p>		

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AN ENZYME

The present invention relates to an enzyme. The present invention also relates to a nucleotide sequence coding for the enzyme.

5

Boos and coworkers in 1981 and 1982 (1, 2) presented evidence for the existence of an enzyme capable of acetylating maltose *via* transfer of the acetyl group from Acetyl-coenzyme A to maltose in *E. coli*. In particular, Boos *et al* (1) observed the formation of acetyl-maltose and acetyl-oligomaltosides after accumulation of maltose or maltooligosides in *E. coli*. They also observed the formation of acetyl-maltose and acetyl-oligomaltosides *in vitro* when maltose or maltotriose, acetyl-coenzyme A and a cytosolic *E. coli* extract were mixed together Boos *et al* (2).

10

Boos *et al* in 1981 stated that the activity responsible for maltose and maltodextrin acetylation was unknown. However, in their further studies of 1982 (2), Feundlieb and Boos named the unknown enzyme "maltose transacetylase" but then said that the function of maltose transacetylase in *E. coli* was unclear.

15

Later Brand and Boos (3) isolated an *E. coli* mutant lacking the gene encoding maltose transacetylase. This mutant enabled them to map the gene at 10.4 min on the *E. coli* linkage map. In addition, they cloned a 3.4 kb DNA fragment containing the gene in a high copy plasmid. Over-expressed maltose transacetylase was then purified to homogeneity from cell free extracts of an *E. coli* strain harbouring the above mentioned plasmid. The enzyme was shown to be a homodimer with two identical subunits of 20 kDa. The K_m (mM) and V_{max} ($\mu\text{mol}/\text{min} \times \text{mg enzyme}$) values of this enzyme for the substrates glucose, maltose and acetyl-coenzyme A were 62 and 200, 90 and 110, and 0.018 and 166 respectively. Maltotriose and other oligosaccharides were found to be acetylated with a rate of 2% of the rate determined for glucose. In addition, Brand and Boos presented the following relative transacetylation rates: glucose 1, maltose 0.55, mannose 0.2, fructose 0.07, galactose 0.04, maltotriose and other malto-oligosaccharides 0.02. Oligosaccharides are saccharides having less than ten sugar units.

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Despite of these findings Brand and Boos did not sequence the enzyme or the nucleotide sequence coding for the maltose transacetylase enzyme.

5 According to a first aspect of the present invention there is provided an enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, wherein the enzyme comprises the amino acid sequence shown as SEQ ID No. 1, or a variant, homologue or fragment thereof.

10 According to a second aspect of the present invention there is provided a recombinant enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, wherein the enzyme comprises the amino acid sequence shown as SEQ ID No. 1, or a variant, homologue or fragment thereof.

15 According to a third aspect of the present invention there is provided a recombinant enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, wherein the enzyme has the amino acid sequence shown as SEQ ID No. 1.

20 According to a fourth aspect of the present invention there is provided a recombinant enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, wherein the recombinant enzyme is immunologically reactive with an antibody raised against a purified recombinant enzyme according to the above-mentioned aspect of the present invention.

25 According to a fifth aspect of the present invention there is provided a nucleotide sequence coding for the enzyme of the present invention or a sequence that is complementary thereto.

30 According to a sixth aspect of the present invention there is provided a nucleotide sequence comprising the sequence shown as SEQ ID No. 2, or a variant, homologue or fragment thereof or a sequence that is complementary thereto.

According to a seventh aspect of the present invention there is provided a nucleotide sequence having the sequence shown as SEQ ID No. 2.

5 According to an eighth aspect of the present invention there is provided a construct comprising or expressing the nucleotide sequence or the enzyme of the present invention.

10 According to a ninth aspect of the present invention there is provided a vector comprising or expressing the construct or the nucleotide sequence or the enzyme according to the present invention.

15 According to a tenth aspect of the present invention there is provided a plasmid comprising or expressing the vector, the construct or the nucleotide sequence or the enzyme according to the present invention.

According to an eleventh aspect of the present invention there is provided a transgenic organism comprising or expressing the plasmid, the vector, the construct or the nucleotide sequence or enzyme according to the present invention.

20 According to a twelfth aspect of the present invention there is provided a modified carbohydrate (preferably starch) prepared by a method comprising or expressing or using the present invention.

25 The enzyme of the present invention may be obtainable from any one of a bacterium, a fungus, an alga, a yeast, or a plant. Preferably, the enzyme is obtainable from *E.coli*.

30 The $\alpha(1,4)$ glucan acetyl-transferase of the present invention is sometimes referred to as Mac. The gene coding for the $\alpha(1,4)$ glucan acetyl-transferase of the present invention is also sometimes referred to as the *mac* gene.

According to a seventh aspect of the present invention there is provided a nucleotide sequence having the sequence shown as SEQ ID No. 2.

5 According to an eighth aspect of the present invention there is provided a construct comprising or expressing the nucleotide sequence or the enzyme of the present invention.

10 According to a ninth aspect of the present invention there is provided a vector comprising or expressing the construct or the nucleotide sequence or the enzyme according to the present invention.

15 According to a tenth aspect of the present invention there is provided a plasmid comprising or expressing the vector, the construct or the nucleotide sequence or the enzyme according to the present invention.

20 According to an eleventh aspect of the present invention there is provided a transgenic organism comprising or expressing the plasmid, the vector, the construct or the nucleotide sequence or enzyme according to the present invention.

25 According to a twelfth aspect of the present invention there is provided a modified carbohydrate (preferably starch) prepared by a method comprising or expressing or using the present invention.

The enzyme of the present invention may be obtainable from any one of a bacterium, a fungus, an alga, a yeast, or a plant. Preferably, the enzyme is obtainable from *E. coli*.

30 The $\alpha(1,4)$ glucan acetyl-transferase of the present invention is sometimes referred to as Mac. The gene coding for the $\alpha(1,4)$ glucan acetyl-transferase of the present invention is also sometimes referred to as the *mac* gene.

Preferably, the enzyme comprises the amino acid sequence shown as SEQ ID No 1, or a variant, homologue or fragment thereof.

Preferably, the enzyme has the amino acid sequence shown as SEQ ID No 1.

5

Preferably, the enzyme is encoded by a nucleotide sequence comprising the nucleotide sequence shown as SEQ ID No 2, or a variant, homologue or fragment thereof or a sequence that is complementary thereto.

10 Preferably, the enzyme is encoded by the nucleotide sequence shown as SEQ ID No 2.

Preferably, the organism is a plant.

15 Preferably, the nucleotide sequence is a DNA sequence.

The enzyme or nucleotide sequence(s) coding for same may be used *in vitro* or *in vivo* in combination with one or more other enzymes or nucleotide sequence(s) coding for same, which enzymes or nucleotide sequence(s) coding for same are preferably prepared by use of recombinant DNA techniques.

20

Thus, according to one aspect of the present invention, an *in vivo* enzymatic modification process can be followed by an *in vitro* enzymatic modification process. In these modification steps, the enzymes used need not necessarily be the same enzymes.

25

The terms "variant", "homologue" or "fragment" in relation to the enzyme include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) amino acid from or to the sequence providing the resultant amino acid sequence has $\alpha(1,4)$ glucan acetyl-transferase activity, preferably having at least the same activity of the enzyme shown as SEQ ID No. 1. In particular, the term "homologue" covers homology with respect to structure and/or function

30

providing the resultant enzyme has $\alpha(1,4)$ glucan acetyl-transferase activity. With respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequence shown as SEQ ID No. 1. More preferably there is at least 95%, more preferably at least 98%,
5 homology to the sequence shown as SEQ ID No. 1.

The terms "variant", "homologue" or "fragment" in relation to the nucleotide sequence coding for the enzyme include any substitution of, variation of, modification of, replacement of, deletion of or addition of one (or more) nucleic acid from or to
10 the sequence providing the resultant nucleotide sequence codes for an enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, preferably having at least the same activity of the enzyme shown as SEQ ID No. 1. In particular, the term "homologue" covers homology with respect to structure and/or function providing the resultant nucleotide sequence codes for an enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity. With
15 respect to sequence homology, preferably there is at least 75%, more preferably at least 85%, more preferably at least 90% homology to the sequence shown as SEQ ID No. 2. More preferably there is at least 95%, more preferably at least 98%, homology to the sequence shown as SEQ ID No. 2.

20 The above terms are synonymous with allelic variations of the sequences.

The term "complementary" means that the present invention also covers nucleotide sequences that can hybridise to the nucleotide sequence of the present invention.

25 The term "nucleotide" in relation to the present invention includes genomic DNA, cDNA, synthetic DNA, and RNA. Preferably it means DNA, more preferably cDNA for the coding sequence of the present invention.

Preferably the nucleotide sequence is not a native nucleotide sequence. In this
30 regard, the term "native nucleotide sequence" means an entire nucleotide sequence that is in its native environment and when operatively linked to an entire promoter with which it is naturally associated, which promoter is also in its native environment.

Thus, the enzyme of the present invention can be expressed by a nucleotide sequence in its native organism but wherein the nucleotide sequence is not under the control of the promoter with which it is naturally associated within that organism.

5 The enzyme of the present invention may be used in conjunction with other enzymes.

Preferably the enzyme is not a native enzyme. In this regard, the term "native enzyme" means an entire enzyme that is in its native environment and when it has been expressed by its native nucleotide sequence.

10

The term "construct" - which is synonymous with terms such as "conjugate", "cassette" and "hybrid" - includes the nucleotide sequence directly or indirectly attached or fused to a promoter. An example of an indirect attachment is the provision of a suitable spacer group such as an intron sequence, such as the *Sh1*-
15 intron or the ADH intron, intermediate the promoter and the nucleotide sequence.

In each case, it is highly preferred that the terms do not cover the natural combination of the gene coding for the enzyme ordinarily associated with the wild type gene promoter and when they are both in their natural environment. One highly preferred
20 embodiment of the present invention therefore relates to the nucleotide sequence of the present invention operatively linked to a heterologous promoter.

The construct may even contain or express a marker which allows for the selection of the genetic construct in, for example, a plant, such as potato, into which it has
25 been transferred. Various markers exist which may be used, such as for example those encoding mannose-6-phosphate isomerase (especially for plants) or those markers that provide for antibiotic resistance - e.g. resistance to G418, hygromycin, bleomycin, kanamycin and gentamycin.

30 The term "vector" includes expression vectors and transformation vectors.

The term "expression vector" means a construct capable of *in vivo* or *in vitro*

expression.

5 The term "transformation vector" means a construct capable of being transferred from one species to another - such as from an *E.coli* plasmid to an *Agrobacterium* to a plant.

The term "tissue" includes tissue and organ, which tissue and organ can be isolated tissue and isolated organ, as well as tissue and organ when within an organism.

10 The term "organism" in relation to the present invention includes any organism that could comprise the nucleotide sequence coding for the enzyme according to the present invention and/or products obtained therefrom, and/or wherein the nucleotide sequence according to the present invention can be expressed when present in the organism.

15

Preferably the organism is a plant.

20 The term "transgenic organism" in relation to the present invention includes any organism that comprises the nucleotide sequence coding for the enzyme according to the present invention and/or the products obtained therefrom, and/or wherein the nucleotide sequence according to the present invention can be expressed within the organism. Preferably the nucleotide sequence is incorporated in the genome of the organism.

25 Preferably the transgenic organism is a plant.

30 Therefore, the transgenic organism of the present invention includes an organism comprising any one of, or combinations of, the nucleotide sequence coding for the enzyme according to the present invention, constructs according to the present invention, vectors according to the present invention, plasmids according to the present invention, cells according to the present invention, tissues according to the present invention, or the products thereof. For example the transgenic organism can

also comprise the nucleotide sequence coding for the enzyme of the present invention under the control of a heterologous promoter. The transgenic organism does not comprise the combination of a promoter and the nucleotide sequence coding for the enzyme according to the present invention, wherein both the promoter and the
5 nucleotide sequence are native to that organism and are in their natural environment.

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Mond theory of gene expression.

10 The promoter could additionally include one or more features to ensure or to increase expression in a suitable host. For example, the features can be conserved regions such as a Pribnow Box or a TATA box. The promoters may even contain other sequences to affect (such as to maintain, enhance, decrease) the levels of expression of the nucleotide sequence of the present invention. For example, suitable other
15 sequences include the *ShI*-intron or an ADH intron. Other sequences include inducible elements - such as temperature, chemical, light or stress inducible elements.

Also, suitable elements to enhance transcription or translation may be present. An example of the latter element is the TMV 5' signal sequence (see Sleat Gene 217
20 [1987] 217-225; and Dawson Plant Mol. Biol. 23 [1993] 97).

Thus, in one aspect, the nucleotide sequence according to the present invention is under the control of a promoter that allows expression of the nucleotide sequence. In this aspect, the promoter may be a cell or tissue specific promoter. If, for
25 example, the organism is a plant then the promoter can be one that affects expression of the nucleotide sequence in any one or more of seed, stem, tuber, sprout, root and leaf tissues.

General teachings of recombinant DNA techniques may be found in Sambrook, J., Fritsch, E.F., Maniatis T. (Editors) Molecular Cloning. A laboratory manual. Second
30 edition. Cold Spring Harbour Laboratory Press. New York 1989.

Even though the enzyme and the nucleotide sequence of the present invention are not disclosed in EP-B-0470145 and CA-A-2006454, those two documents do provide some useful background commentary on the types of techniques that may be employed to prepare transgenic plants according to the present invention. Some of these background teachings are now included in the following commentary.

The basic principle in the construction of genetically modified plants is to insert genetic information in the plant genome so as to obtain a stable maintenance of the inserted genetic material.

Several techniques exist for inserting the genetic information, the two main principles being direct introduction of the genetic information and introduction of the genetic information by use of a vector system. A review of the general techniques may be found in articles by Potrykus (*Annu Rev Plant Physiol Plant Mol Biol* [1991] 42:205-225) and Christou (*Agro-Food-Industry Hi-Tech* March/April 1994 17-27).

Thus, in one aspect, the present invention relates to a vector system which carries the nucleotide sequence or construct according to the present invention and which is capable of introducing the nucleotide sequence or construct into the genome of an organism, such as a plant.

The vector system may comprise one vector, but it can comprise two vectors. In the case of two vectors, the vector system is normally referred to as a binary vector system. Binary vector systems are described in further detail in Gynheung An et al. (1980), *Binary Vectors, Plant Molecular Biology Manual A3*, 1-19.

One extensively employed system for transformation of plant cells with a given promoter or nucleotide sequence or construct is based on the use of a Ti plasmid from *Agrobacterium tumefaciens* or a Ri plasmid from *Agrobacterium rhizogenes* An et al. (1986), *Plant Physiol.* 81, 301-305 and Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208.

Several different Ti and Ri plasmids have been constructed which are suitable for the construction of the plant or plant cell constructs described above.

5 The nucleotide sequence or construct of the present invention should preferably be inserted into the Ti-plasmid between the terminal sequences of the T-DNA or adjacent a T-DNA sequence so as to avoid disruption of the sequences immediately surrounding the T-DNA borders, as at least one of these regions appear to be essential for insertion of modified T-DNA into the plant genome.

10 As will be understood from the above explanation, if the organism is a plant, then the vector system of the present invention is preferably one which contains the sequences necessary to infect the plant (e.g. the *vir* region) and at least one border part of a T-DNA sequence, the border part being located on the same vector as the genetic construct.

15 Furthermore, the vector system is preferably an *Agrobacterium tumefaciens* Ti-plasmid or an *Agrobacterium rhizogenes* Ri-plasmid or a derivative thereof, as these plasmids are well-known and widely employed in the construction of transgenic plants, many vector systems exist which are based on these plasmids or derivatives thereof.

20

In the construction of a transgenic plant the nucleotide sequence or construct of the present invention may be first constructed in a microorganism in which the vector can replicate and which is easy to manipulate before insertion into the plant. An example
25 of a useful microorganism is *E. coli*, but other microorganisms having the above properties may be used. When a vector of a vector system as defined above has been constructed in *E. coli*, it is transferred, if necessary, into a suitable *Agrobacterium* strain, e.g. *Agrobacterium tumefaciens*. The Ti-plasmid harbouring the nucleotide sequence or construct of the invention is thus preferably transferred into a suitable
30 *Agrobacterium* strain, e.g. *A. tumefaciens*, so as to obtain an *Agrobacterium* cell harbouring the nucleotide sequence or construct of the invention, which DNA is subsequently transferred into the plant cell to be modified.

As reported in CA-A-2006454, a large amount of cloning vectors are available which contain a replication system in *E. coli* and a marker which allows a selection of the transformed cells. The vectors contain for example pBR 322, pUC series, M13 mp series, pACYC 184 etc. In this way, the nucleotide or construct of the present invention can be introduced into a suitable restriction position in the vector. The contained plasmid is used for the transformation in *E. coli*. The *E. coli* cells are cultivated in a suitable nutrient medium and then harvested and lysed. The plasmid is then recovered. As a method of analysis there is generally used sequence analysis, restriction analysis, electrophoresis and further biochemical-molecular biological methods. After each manipulation, the used DNA sequence can be restricted and connected with the next DNA sequence. Each sequence can be cloned in the same or different plasmid.

After each introduction method of the construct or nucleotide sequence according to the present invention in the plants the presence and/or insertion of further DNA sequences may be necessary. If, for example, for the transformation the Ti- or Ri-plasmid of the plant cells is used, at least the right boundary and often however the right and the left boundary of the Ti- and Ri-plasmid T-DNA, as flanking areas of the introduced genes, can be connected. The use of T-DNA for the transformation of plant cells has been intensively studied and is described in EP-A-120516; Hoekema, in: The Binary Plant Vector System Offset-drukkerij Kanters B.B., Alblaserdam, 1985, Chapter V; Fraley, et al., Crit. Rev. Plant Sci., 4:1-46; and An et al., EMBO J. (1985) 4:277-284.

Direct infection of plant tissues by *Agrobacterium* is a simple technique which has been widely employed and which is described in Butcher D.N. et al. (1980), *Tissue Culture Methods for Plant Pathologists*, eds.: D.S. Ingrams and J.P. Helgeson, 203-208. For further teachings on this topic see Potrykus (Annu Rev Plant Physiol Plant Mol Biol [1991] 42:205-225) and Christou (Agro-Food-Industry Hi-Tech March/April 1994 17-27). With this technique, infection of a plant may be done on a certain part or tissue of the plant, i.e. on a part of a leaf, a tuber, a root, a stem or another part of the plant.

Typically, with direct infection of plant tissues by *Agrobacterium* carrying the nucleotide sequence of the present invention, a plant to be infected is wounded, e.g. by cutting the plant with a razor or puncturing the plant with a needle or rubbing the plant with an abrasive. The wound is then inoculated with the *Agrobacterium*. The inoculated plant or plant part is then grown on a suitable culture medium and allowed to develop into mature plants.

When plant cells are constructed, these cells may be grown and maintained in accordance with well-known tissue culturing methods such as by culturing the cells in a suitable culture medium supplied with the necessary growth factors such as amino acids, plant hormones, vitamins, etc.

Regeneration of the transformed cells into genetically modified plants may be accomplished using known methods for the regeneration of plants from cell or tissue cultures, for example by selecting transformed shoots using an antibiotic and by subculturing the shoots on a medium containing the appropriate nutrients, plant hormones, etc.

Even further useful teachings on the transformation of plants can be found in Danish patent application No. 940662 (filed 10 June 1994) and/or United Kingdom patent application No. 9702592.8 (filed 7 February 1997).

Reference may even be made to Spngstad *et al* (1995 Plant Cell Tissue Organ Culture 40 pp 1-15) as these authors present a general overview on transgenic plant construction.

In summation, the present invention relates to an enzyme having $\alpha(1,4)$ glucan acetyltransferase activity and a nucleotide coding for same. The present invention also provides a modified carbohydrate (preferably starch) obtainable from use of the same.

The following sample was deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria

Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom.
AB2 1RY on 7 March 1996:

- 5 DH5 α -pMAC3 (which contains a 3.2 kb *EcoRI*-*Pst*I fragment from *E. coli* comprising the *mac* gene).

The deposit number is NCIMB 40789.

- 10 This deposit concerns the plasmid pMAC3.

The following sample was deposited in accordance with the Budapest Treaty at the recognised depositary The National Collections of Industrial and Marine Bacteria Limited (NCIMB) at 23 St. Machar Drive, Aberdeen, Scotland, United Kingdom,
15 AB2 1RY on 7 March 1996:

NF1830-pMAC5 (which contains the *E. coli mac* gene).

The deposit number is NCIMB 40790.

20

This deposit concerns the plasmid pMAC5.

- 25 A highly preferred aspect of the present invention therefore relates to an enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, wherein the enzyme comprises the amino acid sequence shown as SEQ ID No. 1, or a variant, homologue or fragment thereof; and wherein the enzyme is expressed by a nucleotide sequence obtainable from either deposit number NCIMB 40789 or deposit number NCIMB 40790.

- 30 Another highly preferred aspect of the present invention therefore relates to a nucleotide sequence comprising the sequence shown as SEQ ID No. 2, or a variant, homologue or fragment thereof or a sequence that is complementary thereto, and wherein the nucleotide sequence is obtainable from either deposit number NCIMB

40789 or deposit number NCIMB 40790.

The present invention also provides a modified carbohydrate (preferably starch) obtainable from use of this same plasmid.

5

The present invention will now be described only by way of example in which reference is made to the following Figures:

Figure 1 which shows the nucleotide sequence corresponding to SEQ ID No. 2;

10

Figure 2 which shows the amino acid sequence corresponding to SEQ ID No. 1;

Figure 3 which shows a nucleotide sequence comprising the sequence corresponding to SEQ ID No. 2;

15

Figure 4 which is a plasmid map of pMAC1;

Figure 5 which is a plasmid map of pMAC2;

20

Figure 6 which is a plasmid map of pMAC3;

Figure 7 which is a plasmid map of pMAC5;

Figure 8 which is a plasmid map of pMAC8;

25

Figure 9 which is a plasmid map of pMAC9; and

Figure 10 which is a plasmid map of pMAC10.

30

Some details on the Figures are as follows:

Figure 1

Nucleotide sequence corresponding to Seq ID No 2

Figure 2

- 5 Amino acid sequence corresponding to Seq ID No 1
183 amino acids
20073 MW

Figure 4

- 10 Plasmid name: pMAC1
Plasmid size: 7.26 kb
Comments: Insertion of a 4.3 kb *EcoR*I fragment from *lambda* 151 into the *EcoR*I site of pBluescript II SK +.

15 **Figure 5**

Plasmid name: pMAC2
Plasmid size: 7.26 kb
Comments: Insertion of a 4.3 kb *EcoR*I fragment from *lambda* 151 (Kohara collection) into the *EcoR*I site of pBluescript II SK +.

20

Figure 6

Plasmid name: pMAC3
Plasmid size: 7.26 kb
Comments: Deletion of the 1.1 kb *Pst*I fragment from pMAC2.

25

Figure 7

Plasmid name: pMAC5
Plasmid size: 4060 bp
Comments:

- 30 The *E coli mac* gene was amplified with primers:
#B411 (upper primer with *EcoR*I site)
CGG AAT TCC GCC ATG AAG ACA TAC CC

#B412 (lower primer with *Hind*III site)

CAC AAG CTT ATT TTG CAT AAC AGT TGC

using pMAC3 as template.

The 704 bp PCR product was digested with *Eco*R1 and *Hind*III and inserted in

5 pUHE21-2 digested with the same restriction enzymes.

Figure 8

Plasmid Name: pMAC8

Plasmid size: 4935 bp

10 Comments: The *E coli mac* gene was amplified with primers

B 478 CGG GAT CCG AGC ACA GAA AAA GAA AAG ATG (upper
primer with *Bam*HI site)

15 # B 479 AAC TGC AGA TTT TGC ATA ACA GTT GC (lower primer with
*Pst*I site)

and pMAC3 as template. The PCR product was digested with *Bam*HI and *Pst*I and
inserted in pBETP5 digested with the same enzymes.

20

The SBE TP-*mac* fusion was control sequenced with primer # C028

The 35S terminator-*mac* fusion was sequenced with primer # B456 og # C027.

Figure 9

25 Plasmid name: pMAC9

Plasmid size: 9.37 kb

Comments: Insertion of the 2294 bp *Eco*RI fragment (Patatin promoter-SBE TP-*mac* -
35S terminator) from pMAC8 in the *Eco*RI site of pVictor IV Man.

30

Figure 10

Plasmid name: pMAC10

Plasmid size: 9.37 kb

Comments: Insertion of a 2294 bp *EcoRI* fragment (Patatin promoter-SBE TP-mac-
5 35S terminator) from pMAC8 in the *EcoRI* site of pVictor IV Man.

Cloning and sequencing of the *mac* gene from *E. coli*.

Following, initially the teachings of Boos and Brand (3), the *mac* gene was isolated
10 from the 4.3 kb *EcoRI* fragment from λ phage 8C4 (151) from the Kohara collection
(4). The fragment was inserted into the *EcoRI* site of plasmid pBluescript II SK (+)
in both orientations yielding plasmids pMAC1 and pMAC2 (Figures 4 and 5). When
harboured in *E. coli* these plasmids gave rise to highly elevated maltose
acetyltransferase levels indicating that the 4.3 kb *EcoRI* fragment contains the *mac*
15 gene.

In order to localise the *mac* gene on the 4.3 kb *EcoRI* fragment, the 1.1 kb *PstI*
fragment was deleted from plasmid pMAC2. This plasmid construction pMAC3
(Figure 6) also gave rise to increased maltose acetyltransferase levels in strains
20 containing this plasmid, thus demonstrating that the *mac* gene is present on the 3.2
kb *EcoRI-PstI* fragment.

The nucleotide sequence of the 3.2 kb *EcoRI-PstI* insert in pMAC3 was then
determined by automated sequencing on an A.L.F. sequencer. The 3137 bp DNA
25 sequence revealed a 372 bp region of the 3' end of the *E. coli acrB* gene and three
open reading frames potentially encoding proteins of 124, 126, and 183 amino acids
(Figure 3).

In accordance, ³⁵S-methionine labelling experiments with *E. coli* minicells containing
30 pMAC3 showed the synthesis of proteins having molecular weights corresponding to
these sizes.

The 183 codon orf which encodes a protein of a predicted molecular weight of 20073 (Figure 2) is the *mac* gene, since the *E. coli* maltose acetyl-transferase has an estimated subunit molecular weight of 20.000 (3).

5 Over-expression of the Mac enzyme in *E. coli*.

In order to purify the Mac enzyme, the *mac* gene was inserted after an isopropylthiogalactosidase (IPTG) inducible phage T7-promoter A1 in pUHE21-2 to give pMAC5 (Figure 7). Cultures of *E. coli* strain NF1830 (MC1000, recA1, F' lacIq1Z::tm5, a gift from Niels Fiil, University of Copenhagen) harbouring pMAC5
10 was found to have highly elevated levels of maltose acetyltransferase, when expression of the *mac* gene is induced by addition of IPTG to the growth medium.

Growth Conditions

15

A 1 L LB culture of NF1830-pMAC5 supplemented with ampicillin (100 µg/ml) and kanamycin (25 µg/ml) was grown at 37°C with vigorous shaking until the A600 reached 0.7. IPTG was added to a final concentration of 2mM and growth was continued for four hours. The cells were harvested by centrifugation (10 min. at 4
20 000 x g) and washed by resuspension in 200 ml 0.9% NaCl. The cell pellet was then resuspended in 250 ml 20 mM potassium phosphate pH 7.5 containing 0.4 mM PMSF, 0.4 mg/ml pepstatin and 1.6 mM EDTA. The suspension was sonicated 5 x 1 min. using a Vibra Cell VC 600 with a 19 mm High Gain Horn and extender (all from Sonics and Materials Inc., USA). The homogenate was clarified by
25 centrifugation for 60 min. at 90 000 x g at 4°C and subsequent filtration through a 0.22 µm filter.

Purification of Recombinant Mac

The resulting crude extract was applied to a Q-Sepharose 26/10 column (Pharmacia Biotech) equilibrated with 20 mM potassium phosphate pH 7.5 (hereinafter called "buffer A") at a flow rate of 2 ml/min. The column was washed with 300 ml of buffer A and the bound protein was eluted by applying a 0 to 0.3 M NaCl linear gradient in buffer A (300 ml). The fractions containing enzyme activity were pooled and applied to a 8 ml Affi-Gel Blue (Biorad) column (16 mm x 26 cm) equilibrated with buffer A at a flow rate of 1 ml/min. The column was washed with 50 ml of the same buffer containing 0.4 M NaCl. The enzyme was then eluted with the same buffer containing 2 M NaCl. The active pool was dialysed overnight against buffer A and subsequently concentrated to approximately 3 ml in a Centriprep-30 (Amicon). This fraction was applied to a 6 ml Acetyl-coA-Minileak column equilibrated with buffer A at a flow rate of 0.3 ml/min. This affinity resin was made by coupling 200 mg of Acetyl-coA to 5 g (dry weight) of Minileak High (Kem-En-Tek, Denmark) in 10 ml of 1 M NaCO₃ pH 11 for 20h at room temperature. The column was washed with 20 ml of buffer A. It was then turned upside down and the pure enzyme was eluted in less than 20 ml with buffer A containing 0.5 M NaCl.

The purification of the maltose acetyltransferase to homogeneity was achieved after three chromatographic steps. From 1l culture we were able to get 5.8 mg pure Mac. The yield was 29% and the enzyme was purified 80-fold. The purity of the enzyme was assessed both by SDS-PAGE and mass spectrometry. The latter revealed a molecular mass of 19,982 Da.

Determination of enzyme concentration and activity

The concentration of pure Mac solutions was estimated spectrophotometrically at 280 nm using an extinction coefficient of 0.66 as determined from the amino acid composition of Mac according to (5). The acetyl-transferase activity of Mac was assayed spectrophotometrically according to a modified Alpers' assay (6). A Perkin Elmer Lambda 18 spectrophotometer was used. The assay mixture of a total volume of 1 ml contained a 50 mM potassium phosphate, 2 mM EDTA buffer at pH 7.5, 100 μ l of maltose 1M, 100 μ l of Acetyl-coA 0.4 mM, 10 μ l 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) 40 mM dissolved in methanol and 10 μ l enzyme. The reaction was started by the addition of enzyme or maltose and was monitored at 412 nm at 25°C. One activity unit was defined as the amount of enzyme that produced an increase in absorbance of 1 per minute at 25°C. An extinction coefficient of 13 600 M⁻¹ x cm⁻¹ was used for DTNB in order to calculate the consumption of acetyl coenzyme A.

N-terminal sequencing of Recombinant Mac

N-terminal sequencing of pure Mac was performed using an Applied Biosystems 476A protein sequencer. One nanomole of protein was desalted by RP-HPLC on a C2 column (4.6/30) prior to loading onto the sequencer. The N-terminal sequence of Mac was determined up to residue 48 and was in complete concordance with the nucleotide sequence of the *mac* gene (Figure 1). Furthermore, the N-terminal methionine residue was not present on the mature protein (Figure 2).

Production of polyclonal antibodies against Recombinant Mac

Rabbits were immunised subcutaneously at 2-week intervals during 6 weeks and at 4-week intervals thereafter with 90 μ g of pure protein emulsified (1:1, vol/vol) with Freund's adjuvant. Antisera were tested against Mac in immunoblots and were found highly specific.

Characterisation and activity profile of recombinant Mac

Mass spectrometry studies indicated that Mac may be a trimer.

- 5 The isoelectric point of Mac was determined by isoelectric focusing on a PhastGel IEF 4-6.5 (Pharmacia) and was found to be 5.7.

10 The pH profile of Mac was investigated between pH 5 and 8.5 at a 100 mM maltose concentration in 50 mM buffers containing 100 mM NaCl. Under these conditions, the pH optimum was 7.7.

15 The pH stability of Mac was examined at 25°C between pH 3.0 and 10.0. Mac was instantaneously inactivated at pH 3.0 but was stable between pH 4.0 and 10.0 for at least six hours.

20 The thermostability of Mac was investigated at pH 7.5 between 40 and 70°C. After incubation for four hours at 40°C and 50°C, the remaining activity of Mac was 100% and 75%, respectively. Its half-life was 70 min, and 22 min at 60°C and 70°C, respectively.

25 The substrate preference of Mac towards the carbohydrate acetyl-acceptor substrate was investigated by measuring the initial rate of the acetylation of various carbohydrates (used at 50 and 100 mM concentrations) following the procedure described in "Determination of enzyme concentration and activity". The results are presented in Tables 1, 2 and 3. Among the monosaccharides tested, glucose was the best substrate and among the disaccharides tested, maltose and isomaltose were the best substrates.

Table 1. Comparison of the relative activity of Mac towards various monosaccharides as acetyl-acceptors.

Substrate (100 mM)	Relative Activity (% of activity on glucose)
Glucose	100
Mannose	38
Fructose	17
Galactose	0.9

10

Table 2. Comparison of the relative activity of Mac towards various disaccharides as acetyl-acceptors.

Substrate (100 mM)	Relative Activity (% of activity on maltose)
Maltose (α -glucose(1,4) α -glucose)	100
Isomaltose (α -glucose(1,4) α -glucose)	110
Lactose (β -galactose β -(1,6) α -glucose)	0.4
Sucrose (α -glucose α -(1,4) β -fructose)	0.4
Cellobiose (β -glucose β -(1,4) β -glucose)	0

15

20

Table 3. Comparison of the relative activity of Mac towards various maltooligosaccharides as acetyl-acceptors.

Substrate (50 mM)	Relative Activity (% of activity on maltose)
Maltose	100
Maltotriose	7.5
Maltotetraose	0.5
Maltopentaose	0.9
Maltohexaose	1.2
Maltoheptaose	1.1

Kinetic studies

Kinetic studies of Mac catalysed acetylation reactions revealed that the K_m for the acceptor substrate is in the mM range whereas it is in the μM range for acetyl-coenzyme A. Thus, Mac has about a 1000 fold more affinity for acetyl-coenzyme A than for the acceptor.

NMR studies

1H -NMR structure determination of the products of acetylation of glucose and maltose by Mac was investigated.

In order to investigate the substrate regio-specificity of Mac regarding the acetylation site of the acceptor substrate, we prepared milligram amounts of acetylated glucose and maltose by incubating 10 mg of glucose or maltose with *E. coli* Mac and 1 mg acetyl-coenzyme A in phosphate buffer at pH 7.5 for 48 hours. Additional aliquots

of 1 mg acetyl-coenzyme A were added during the incubation. The reaction products were separated by thin layer chromatography and the acetylated glucose and maltose were isolated from the chromatogram and freeze dried. The structures of these acetylated sugars were determined by ¹H-NMR. Glucose was only acetylated at the C6 position, and maltose was acetylated at the C6 position of its non-reducing glucose moiety. These results reveal that Mac acetylates hexoses at their C6 position.

Activity of the SBE-Mac fusion in *E. coli*.

Because the 27 amino acid SBE portion of the SBE-Mac fusion in pMAC9 and pMAC10 described below may interfere with the acetyltransferase activity, the SBE-Mac fusion was inserted in the *E. coli* expression vector pAL781 (Invitrogene, San Diego, USA) in order to over-express the fusion enzyme in *E. coli* and analyse the activity. A comparison of the highly over-expressed SBE-Mac fusion and the purified wild type Mac enzyme on SDS gels showed that the fusion migrated slightly slower due to the 27 amino acid extension. Moreover, the fusion retained the ability to use maltose as a substrate for acetylation. Thus, the fusion enzyme appears to be intact and is fully active in *E. coli*. Therefore, it may be assumed, that the SBE-Mac fusion enzyme will be active in potatoes.

IN VIVO MODIFICATION OF STARCH IN POTATO

General teachings on potato transformation may be found in our copending patent applications PCT/EP96/03053, PCT/EP96/03052 and PCT/EP94/01082 (the contents of each of which are incorporated herein by reference).

For the present studies, the following protocol was adopted.

Construction of plasmids for the expression of the *E coli mac* gene in potato.

The *E coli mac* gene was amplified with primers:

5 5'-CGG GAT CCG AGC ACA GAA AAA GAA AAG ATG-3' (upper primer with *Bam*HI site)

and

10 5'-AAC TGC AGA TTT TGC ATA ACA GTT GC-3' (lower primer with *Pst*I site)

and pMAC3 as template.

The PCR product was digested with *Bam*HI and *Pst*I and inserted in pBETP5 (see
15 PCT patent application No. WO 94/24292, the contents of which are incorporated
herein by reference) digested with the same enzymes yielding pMAC8. Thereby, the
mac gene is inserted in an expression cassette that provides tuber specific expression
from a patatin promoter and transcription termination at a CaMV 35S terminator.
Moreover, the Mac enzyme is fused to 102 amino acids of the N-terminus of the
20 potato starch branching enzyme including a 75 amino acid transit peptide that directs
the *mac* gene product to the potato tuber amyloplasts. Upon import to the amyloplast
the 75 amino acid transit peptide is cleaved off, to give a Mac fusionprotein that has
the 27 amino acids from the mature starch branching enzyme N-terminus. The 2294
bp *Eco*RI expression cassette was isolated from pMAC8 and inserted in the *Eco*RI site
25 of the plant transformation vector pVictor IV Man (see PCT patent application No.
WO 94/24292 and British patent application No. 951443.8, the contents of each of
which are incorporated herein by reference) giving plasmids pMAC9 and pMAC10
(Figures 9 and 10, respectively).

Preparation of potato minitubers

A segment containing the nodium - i.e. a segment taken from 2 mm above and 5 mm below the nodium - was cut from *in vitro* grown potato plants or mannose selected shoots (for mannose selection see our earlier patent applications WO 93/05163 and/or WO 94/20627). The leaf was removed from the nodium segment, and the segment was placed vertically on agar plates with MS medium (Sigma) supplemented with 60 g sucrose/l and 2 mg 6-benzyl-aminopurine/l. The nodium segments were grown for 7 days at 20°C with a 16 hour light period and an 8 hour dark period. Subsequently, the plates were wrapped in alu-foil and placed in the dark at 20°C. The minitubers were harvested after about 28 days and applied for western analysis in order to detect Mac expression.

Expression of the SBE-Mac fusion in potato minitubers

Potato minitubers transformed with the pMAC9 or pMAC10 constructs were examined by Western analysis for expression of the *E. coli* mac gene with antibodies raised towards the *E. coli* maltose acetyltransferase. The analysis clearly demonstrated that 3 out of 5 MAC9 minitubers and 5 out of 7 MAC10 minitubers gave a distinct expression of the *E. coli* maltose acetyltransferase. The positive minitubers expressed a 209 amino acid SBE-Mac fusion that co-migrates with a similar construction expressed in *E. coli*. These results indicate that the 75 amino acid SBE transit peptide, that was originally fused to the 209 amino acid SBE-Mac fusion, has been removed from the SBE-fusion. Furthermore, this implies that the transit peptide was correctly processed by the signal peptidase in the amyloplast membrane, and that the SBE-Mac fusion has been directed to the amyloplast.

Immunoblots on potato tuber extracts

0.5 ml potato protein extract was precipitated with 20% TCA for 30 min on ice. Protein precipitates were recovered after centrifugation and resuspended in 50 μ l of
5 SDS-PAGE sample buffer. 25 μ l were subsequently loaded onto 15 % polyacrylamide gels. After electrophoresis proteins were transferred onto Problot PVDF membranes by semi-dry blotting. For immunodetection Mac antiserum was diluted 1:2 000 and secondary antibody was coupled to alkaline phosphatase.

10 In accordance with the Western Blot analysis of the minitubers described above, the western analysis of the transgenic tubers clearly demonstrated that the 209 a SBE-Mac fusion is expressed in the tubers.

Analysis of potato tubers for Mac activity

15 Potato tubers of comparable sizes were chosen and cut into pieces and homogenised in extraction buffer and Dowex (1%, w/vol) using a mortar and pestle or an electric blender. 5 ml extraction buffer (50 mM potassium phosphate pH 7.5, 2 mM EDTA, 0.5 mM PMSF) was used per gram potato. The mixture was allowed to stand on ice
20 for 30 min and the insoluble material was removed by centrifugation. Protein concentration was measured using the BCA reagent (Pierce).

Mac activity was measured in duplicates or triplicates as follows: 0, 50, 100 or 200 μ l potato extract, 10 μ l of 1 mM acetyl-coenzyme A, 25 μ l of 1 M glucose and assay
25 buffer (50 mM potassium phosphate, 2 mM EDTA, pH 7.5) were mixed per microtiter plate well to give a total volume of 250 μ l. The reaction was started by the addition of acetyl-coenzyme A. After 10 min. reaction at room temperature, 25 μ l of freshly made 4 mM DTNB was added and A_{405} was measured immediately. Two wells were prepared for each single assay, one with glucose and one without.
30 Activity was calculated by subtracting the absorbance of the well without glucose (background absorbance) from that of the well with glucose.

Relatively high levels of Mac activity could be measured in eight out of nine transgenic tubers. Some of the tubers had a Mac activity that was 15 to 20 fold above the almost negligible activity found in non-transformed tubers.

5 Viscometric studies

Samples of starch obtained from tubers of non-transformed potatoes and from transformed potatoes according to the present invention were analysed by viscoamylograph of an aqueous suspension using a Newport Scientific Rapid Visco
10 Analyser 3C. The results showed that the starch from the transformed potatoes had a different viscometric profile to the starch from the non-transformed potato.

DSC studies

15 Samples of starch obtained from tubers of non-transformed potatoes and from transformed potatoes according to the present invention were analysed by differential scanning colometry (using a 10% w/w aqueous starch suspension). The samples were heated from 20 to 100°C at a velocity of 10°C per minute. The results showed that the starch from the transformed potatoes had a different enthalpy to the starch from
20 the non-transformed potato. We additionally found a difference in gelatinisation temperature for the transformed potatoes compared to the starch from the non-transformed potatoes.

Other modifications of the present invention will be apparent to those skilled in the
25 art.

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SEQUENCES

SEQUENCE ID NO 1

5 Amino acid sequence

MSTEKEKMIAGELYRSADETLSDRLRARQLIHRYNHSLAEHTLRQQIL 50

10

ADLFGQVTEAYIEPTFRCDYGYNIFLGNNFFANFDCVMLDVCPIRIGDNC 100

MLAPGVHIYTATHPIDPVARNPGAELGKPVITGNNVWIGGRAVINPGVTI 150

GDNVVVASGAVVTKDVPDNNVVGGNPARIIKKL 183

15 SEQUENCE ID NO 2

Nucleotide sequence

	ATGAGCACAG	AAAAAGAAAA	GATGATTGCT	GGTGAGTTGT
20	ATCGCTCGGC	AGATGAGACG	TTATCTCGCG	ATCGCCTGCG
	CGCTCGTCAG	CTTATTCACC	GATACAATCA	TTCCCTGGCG
	GAAGAGCACA	CATTACGCCA	GCAAATTCTC	GCTGATCTAT
	TCGGTCAGGT	GACAGAGGCT	TATATTGAGC	CAACGTTTCG
	CTGTGACTAT	GGCTATAACA	TTTTTCTCGG	TAATAATTTT
25	TTCCGCAACT	TCGATTGCGT	GATGCTTGAT	GTCTGCCCTA
	TTCCGATCGG	TGATAACTGT	ATGTTGGCAC	CAGGCGTTCA
	TATCTACACG	GCAACACATC	CCATCGACCC	TGTAGCACGT
	AATAGCGGTG	CTGAACGGG	GAAACCCGTC	ACCATCGGTA
	ATAACGTCTG	GATTGGCGGA	CGCGCGGTCA	TTAACCCTGG
30	TGTGACCATT	GGTGATAACG	TCGTGGTAGC	CTCAGGTGCA
	GTTGTCACAA	AASATGTCCC	GGACAACGTT	GTCGTGGGCG
	GTAATCCAGC	CAGAATAATT	AAAAAATTGT	AA

SEQUENCE I D NO 3

Nucleotide sequence

Complete nucleotide sequence of the 3.2 kb *EcoRI-PstI* fragment in pMAC3

5	GAATTCGCCA AAGACTTGAT GGATAAAGAA GGTAAGGTC TGATTGAAGC GACGCTTGAT	60
	GCGGTGCGGA TCGGTTTACG TCCGATCCTG ATGACCTCGC TGGCGTTTAT CCTCGGCGTT	120
	ATGCCGCTGG TTATCAGTAC TGGTGCTGGT TCCGGCGCGC AGAACGCAGT AGGTACCGGT	180
	GTAATGGGCG GGATGGTGAC CGCAACGGTA CTGGCAATCT TCTTCGTTCC GGTATTCCTT	240
10	GTGGTGGTTC GCCGCCGCTT TAGCCGCAAG AATGAAGATA TCGAGCACAG CCATACTGTC	300
	GATCATCATT GATACAACGT GTAATCACTA AGGCCGCGTA AGCGGCCTTT TTTATGCATA	360
	ACCTACGAAC ATTAAGGAGT AATTGAACCA CCAACTCAGG ATCTCATACG AAAACCGATA	420
	TTAACCACGG ATAAAATTCA TAAAAAATAC TGATTGTTAG TTAATTTATA TTAAGTAGCG	480
	CTAATAGATT TAATAATCCA TAATCATTTA GAGGCTATTC TTAATTATTT GCGGTAATTC	540
15	TTTATTCATT CCTCGGTTAT TACGTCATAT TCAGAGCAAT CCTGGTATTA GTGTCACCAA	600
	TTTCATCTGG CGATAATCCT GAAATGTTAT GAATAGTTCG AGCAAACTGC TTTTACCTGC	660
	TGCGGGTTAG TGCTAGTATG AAAAAGTGAG TCCTGTCCCG CTTCTTCCT AATTGTAATT	720
	TTTCGTAATA ATGCGATGAA AACCTGCAAA GAGTGGCTTA TAGTTAAGCT AACAAACGAG	780
	AGGGCAAGTC CAGGTCAGTA AGTTTTTCC ATCCCGAAAAG GTGTCCGTTA GTTCAACCGC	840
20	TAAGAAGGGG ACGCGTTATG GATGAATACT CACCCAAAAG ACATGATATC GCACAGCTTA	900
	AGTTTCTCTG TGAAACCCTG TATCATGACT GCCTTGCAAA CCTTGAAGAA AGCAATCATG	960
	GCTGGGTAAT CGACCCAACC TCGGCGATCA ACCTCCAGTT GAATGAACTG ATTGAGCATA	1020
	TTGCGACCTT CGCACTTAAT TACAAAATTA AGTATAATGA AGACAATAAG CTCATTGAGC	1080
	AGATCGACGA ATATCTGGAT GACACCTTTA TGTGTTCAG TAGTTATGGT ATTAATATGC	1140
25	AGGATCTTCA GAAATGGCGG AAGTCAGGTA AHCGACTATH CCGTTGTTTT GTCAATGCGA	1200
	CGAAAGAGAA TCCTGCGAGT TTATCTTGT AGAATTATTA CAACCATAGG TAGAAGTATG	1260
	TCCGAAAAAC CTTTAACGAA AACCGATTAT TTAATGCGTT TACGTCGTTG CCAGACAATT	1320
	GACACGCTGG AGCGGTTTAW TCGAGAAAAA TAAATACGAA TTATCAGATA ATGAACTGGC	1380
	GGTATTTTAC TCAGCCGCGAG ATCACC GCCT CGCCGAATTG ACCATGAATA AACTGTACGA	1440
30	CAAGATCCCT TCCTCAGTAT GGAATTTTAT TCGTAATAA ATAATTGCT TTCGGAGCTA	1500
	TAACCGGCTG TTTATTAAGA ATTTTATACT TTTTCGCCAT GAAGACATAC CCTATGTGAT	1560
	CTTTATCACA CAGATGTAAT GGGAACGTTT TCTTCACTGA CTTTTGCTCT TACTGTGTG	1620
	CCGCATTTTC AGCAACCGGA GTCAGTAATG AGCAGAGAAA AAGAAAAGAT GATTGCTGGT	1680
	GAGTTGTATC GCTCGGCAGA TGAGACGTTA TCTCGCATC GCCTGCGCGC TCGTCAGCTT	1740
35	ATTCACCGAT ACAATCATTC CCTGGCGGAA GAGCACACAT TACGCCAGCA AATTCTCGCT	1800
	GATCTATTCG GTCAGGTGAC AGAGGCTTAT ATTGAGCCAA CGTTTCGCTG TGAATATGGC	1860
	TATAACATTT TTCTCGGTAA TAATTTTTTC GCCAATTCTG ATTGCGTGAT GCTTGATGTC	1920
	TGCCCTATTC GCATCGGTGA TAACTGTATG TTGGCACCAG GCGTTCATAT CTACACGGCA	1980
	ACACATCCCA TCGACCCTGT AGCACGTAAT AGCGGTGCTG AACTGGGGAA ACCCGTCACC	2040
40	ATCGGTAATA ACGTCTGGAT TGGCGGACGC GCGGTCATTA ACCCTGGTGT GACCATGGT	2100
	GATAACGTCG TGGTAGCCTC AGGTGCGATT GTCACAAAAG ATGTCCCGGA CAACGTTGTC	2160
	GTGGGCGGTA ATCCAGCCAG AATAATTAA AAATTGTAAT CGGTTTTTCG CAACTGTTAT	2220
	GCAAAATTGT GGTAGATCTG TTAATTCCTC TCTACTATTC CCACGTTAAA ATAGGGTGTT	2280
	CCCTGGAAAG TTGCAGATAC CACGAAGGCA AACGATGACC GAAATACAAC GCCTGCTGAC	2340
45	CGAAACGATT GAGTCTCTGA ATACCCGCGA AAAACGCGAC AACAAACCCC GCTTTAGTAT	2400

CAGTTTTATC CGTAAACATC CGGGGCTGTT TATCGGTATG TACGTTGCTT TTTTGGCCAC 2460
 CCTGGCGGTG ATGTTGCAGT CCGAAACGCT GTCAGGCTCT GTCTGGCTAC TGGTTGTATT 2520
 ATTTATCCTG CTTAATGGTT TCTTCTTTT CGATGTCTAC CCACGCTACC GCTATGAAGA 2580
 TATCGACGTG CTGGATTTC GCGTTTGCTA TAACGGCGAA TGGTACAACA CGCGCTTTGT 2640
 5 ACCTGCCGCG CTGGTTGAAG CCATCTTGAA CTCTCCGTGT CGCGGATGTT CATAAGGAAC 2700
 AACTGCAAAA AATGATCGTC CGTAAAGGTG AACTGTCTTT TTACGATATT TTTACCTCS 2760
 TCGCGCCGAA TCAACATCTT AAGTTAGGT TACATACCAG GCGTAAAGCT CTGCGCCTGG 2820
 TCAAATGACA ATGATCGTTT CCACCCATCA CTTCATGAAA TACCAGCTCT ACCTCCTTAT 2880
 CTCCAGCCAG CCTTTTCCA CAATCAGATA TACTTCCCT AACTGTGTT AATAAGGATA 2940
 10 TGCTGGTGAG AACACGACAT CTGGTCGGCC TTATTCGGG AGTACTGATT CTTTCAGTAT 3000
 TGCTGCCTGT CGGCTTAAGC ATCTGGCTGG CCCATCAGCA GGTAGAAACA TCGTTTATTG 3060
 AAGAGCTGGA TACCTATTCC TCCCGCGTCG CTATTCGAGC CAATAAGGTG GCGACACAAG 3120
 GGAAAGATGC GCTGCAG 3137

15 SEQUENCE ! D NO 4

Complete nucleotide sequence of the 3.2 kb *EcoRI*-*PstI* fragment in pMAC3. The *Mac* enzyme amino acid sequence is also shown below the *mac* gene coding sequence.

20 GAATTCGCCAAAGACTTGATGGATAAAGAAGGTAAAGGTCTGATTGAAGCGACGCTTGAT 60
 GCGGTGCGGATGCGTTTACGTCCGATCCTGATGACCTCGCTGGCGTTTATCCTCGGCGTT 120
 ATGCCGCTGGTTATCAGTACTGGTCTGGTTCGGCGCGCAGAACGCAGTAGGTACCGGT 180
 GTAATGGGCGGGATGGTGACCGCAACGGTACTGGCAATCTTCTTCGTTCGGTATTCTTT 240
 GTGGTGGTTCCGCCCGCTTTAGCCGAAGAATGAAGATATCGAGCAGCCATACTGTC 300
 25 GATCATCATTGATACAACGTGTAATCACTAAGGCCGCTAAGCGGCTTTTTTATGCATA 360
 ACCTACGAACATTAAGGAGTAATTGAACCACCAACTCAGGATCTCATACGAAAACAGTA 420
 TTAACCACGGATAAAATTCATAAAAAATACTGATTGTTAGTTAATTTATTAAGTAGCG 480
 CTAATAGATTTAATAATCCATAATCATTTAGAGGCTATTCTTAATTTTGGCGTAATTC 540
 TTTATTCATTCCTCGGTTATTACGTCATATTCAGAGCAATCCTGGTATTAGTGTACCAA 600
 30 TTTCTCTGGCGATAATCCTGAAATGTTATGAATAGTTCGAGCAAACGCTTTTACCTGC 660
 TGCGGGTTAGTGCTAGTATGAAAAAGTGAGTCTGTCCCGCTTCCTTCTAATTGTAATT 720
 TTTCTGAATAATGCGATGAAAACCTGCAAAGAGTGGCTTATAGTTAAGCTAACAAACGAG 780
 AGGGCAAGTCCAGGTCAGTAAGTTTTTCCATCCCGAAAGGTGTCCGTTAGTTC AACCGC 840
 TAAGAAGGGGACGCGTTATGGATGAATACTCACCCAAAAGACATGATATCGCACAGCTTA 900
 35 AGTTTCTCTGTGAAACCCTGTATCATGACTGCCTTGCAAACCTGAAGAAAGCAATCATG 960
 GCTGGGTAAACGACCCAACCTCGGCGATCAACCTCCAGTTGAATGAAGTATTGAGCATA 1020
 TTGCGACCTTCGCACTTAATTACAAAATTAAGTATAATGAAGACAATAAGCTCATTGAGC 1080
 AGATCGACGAATATCTGGATGACACCTTTATGTTGTTAGTAGTTATGGTATTAATATGC 1140
 AGGATCTTCAGAAATGGCGGAAGTCAGGTAHCGACTATHCCGTTGTTTTGTCAATGCGA 1200
 40 CGAAAGAGAATCCTGCGAGTTTATCTTGTTAGAATTATTACAACCATAGGTAGAAGTATG 1260
 TCCGAAAACCTTTAACGAAAACCGATTATTTAATGCGTTTACGTCGTTGCCAGACAATT 1320
 GACACGCTGGAGCGGTTTAWTCGAGAAAAATAAATACGAATTATCAGATAATGAACTGGC 1380
 GGTATTTTACTCAGCGCGAGATCACCGCTCGCCGAATTGACCATGAATAAACTGTACGA 1440
 CAAGATCCCTTCCTCAGTATGGAAATTTATTCGCTAATAAATAATTGCTTTTCGAGCTA 1500
 45 TAACCGGCTGTTTATTAAGAATTTTATACTTTTTCGCCATGAAGACATACCCTATGTGAT 1560

	CTTTATCACACAGATGTAATGGGAACGTTCTCTTCACTGACTTTTCGTCTTACTGTGTG	1620
	CCGCATTTTCAGCAACCGGAGTCAGTAATGAGCACAGAAAAAGAAAGATGATTGCTGGT	1680
	M S T E K E K M I A G	
	GAGTTGTATCGCTCGGCAGATGAGACGTTATCTCGCGATCGCCTGCGCGCTCGTCAGCTT	1740
5	E L Y R S A D E T L S R D R L R A R Q L	
	ATTCACCGATAACAATCATTCCCTGGCGGAAGAGCACACATTACGCCAGCAAATTCCTCGCT	1800
	I H R Y N H S L A E E H T L R Q Q I L A	
	GATCTATTCGGTCAGGTGACAGAGGCTTATATTGAGCCAACGTTTCGCTGTGACTATGGC	1860
	D L F G Q V T E A Y I E P T F R C D Y G	
10	TATAACATTTTTCTCGGTAATAATTTTTTCGCCAACTTCGATTGCGTGATGCTTGATGTC	1920
	Y N I F L G N N F F A N F D C V M L D V	
	TGCCCTATTCGCATCGGTGATAACTGTATGTTGGCACCAGGCGTTCATATCTACACGGCA	1980
	C P I R I G D N C M L A P G V H I Y T A	
	ACACATCCCATCGACCTGTAGCACGTAATAGCGGTGCTGAACTGGGGAAACCCGTCACC	2040
15	T H P I D P V A R N S G A E L G K P V T	
	ATCGGTAATAACGTCCTGGATTGGCGGACGCGCGGTCAATACCCCTGGTGTGACCATTTGGT	2100
	I G N N V W I G G R A V I N P G V T I G	
	GATAACGTCGTGGTAGCCTCAGGTGCAGTTGTCACAAAAGATGTCCCGGACAACGTTGTC	2160
	D N V V V A S G A V V T K D V P D N V V	
20	GTGGGCGGTAATCCAGCCAGAATAATTAATAATTGTAATCGGTTTTTCGCAACTGTTAT	2220
	V G G N P A R I I K K L	
	GCAAAATTGTGGTAGATCTGTTACTTCCCTCTACTATTCCCACGTTAAAATAGGGTGT	2280
	CCCTGGAAAGTTGCAGATACCACGAAGGCAACGATGACCGAAATACAACGCCTGCTGAC	2340
	CGAAACGATTGAGTCTCTGAATACCCGCGAAAAACGCGACAACAAACCCGCTTTAGTAT	2400
25	CAGTTTTATCCGTAACATCCGGGGCTGTTTATCGGTATGTACGTTGCTTTTTTGGCAC	2460
	CCTGGCGGTGATGTTGCAGTCCGAAACGCTGTCAGGCTCTGTCTGGCTACTGGTTGTATT	2520
	ATTTATCCTGCTTAATGGTTTCTTCTTTTCGATGTCTACCCACGCTACCGCTATGAAGA	2580
	TATCGACGTGCTGGATTTCCGCGTTTGCTATAACGGCGAATGGTACAACACGCGCTTTGT	2640
	ACCTGCCGCGCTGGTTGAAGCCATCTTGAACCTCCGTGTCGCGGATGTTCAATAAGGAAC	2700
30	AACTGCAAAAAATGATCGTCCGTAAGGTGAACTGTCTTTTACGATATTTTACCCTCS	2760
	TCGCGCCGAATCAACATCTTAAGTTAGGGTTACATACCAGGCGTAAAGCTCTGCGCCTGG	2820
	TCAAATGACAATGATCGTTCCACCCATCACTTCATGAAATACCAGCTCTACCTCCTTAT	2880
	CTCCAGCCAGCCTTTTTCCACAATCAGATATACTTTCCCTACACTGTGTTAATAAGGATA	2940
	TGCTGGTGAGAACACGACATCTGGTCGGCCTTATTTCCGGGAGTACTGATCTTTTCAAT	3000
35	TGCTGCCTGTGGCTTAAGCATCTGGCTGGCCATCAGCAGGTAGAAACATCGTTTATTG	3060
	AAGAGCTGGATACCTATTCCTCCCGCTCGCTATTCGAGCCAATAAGGTGGCGACACAAG	3120
	GGAAAGATGCGCTGCAG	3137

Address of agent or the reference number	PC 375 1 0TH	International application N
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>12613</u> line <u>31608</u>	
B. IDENTIFICATION OF DEPOSIT	
Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of Depository Institution The National Collections of Industrial and Marine Bacteria Limited (NCIMB)	
Address of Depository Institution (including postal code and country) 23 St Machar Drive Aberdeen AB2 1RY United Kingdom	
Date of deposit 7 March 1996	Accession Number NCIMB 40789
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will only be made available either until the publication of the mention of the grant of the patent or after twenty years from the date of filing if the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	
For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer Mrs. T. Bröcker-Tazelaar	Authorized officer

Applicant's or agent's file reference number	PL 376 1 CTH	International application
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INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 136a)

A. The indications made below relate to the microorganism referred to in the description on page <u>13</u> line <u>12 - 21</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet: <input type="checkbox"/>	
Name of depositary institution The National Collections of Industrial and Marine Bacteria Limited (NCIMB)	
Address of depositary institution (including postal code and country) 23 St Machar Drive Aberdeen AB2 1RY United Kingdom	
Date of deposit 7 March 1996	Accession Number NCIMB 40790
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
In respect of those designations in which a European patent is sought, and any other designated state having equivalent legislation, a sample of the deposited microorganism will only be made available either until the publication of the mention of the grant of the patent or after twenty years from the date of filing if the application has been refused or withdrawn or is deemed to be withdrawn, only by the issue of such a sample to an expert nominated by the person requesting the sample. (Rule 28(4) EPC)	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g. "Accession Number of Deposit")	
For receiving Office use only	For International Bureau use only
<input checked="" type="checkbox"/> This sheet was received with the international application	<input type="checkbox"/> This sheet was received by the International Bureau on:
Authorized officer: Mrs. T. Bröcker-Tetzlaff	Authorized officer:

CLAIMS

1. An enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, wherein the enzyme comprises the amino acid sequence shown as SEQ ID No. 1, or a variant,
5 homologue or fragment thereof.
2. A recombinant enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, wherein the enzyme comprises the amino acid sequence shown as SEQ ID No. 1, or a variant, homologue or fragment thereof.
10
3. A recombinant enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity, wherein the enzyme has the amino acid sequence shown as SEQ ID No. 1.
4. A recombinant enzyme having $\alpha(1,4)$ glucan acetyl-transferase activity,
15 wherein the recombinant enzyme is immunologically reactive with an antibody raised against a purified recombinant enzyme according to claim 3.
5. A nucleotide sequence coding for the enzyme of any one of claims 1 to 4 or a sequence that is complementary thereto.
20
6. A nucleotide sequence according to claim 5, wherein the nucleotide sequence is a DNA sequence.
7. A nucleotide sequence comprising the sequence shown as SEQ ID No. 2, or
25 a variant, homologue or fragment thereof or a sequence that is complementary thereto.
8. A nucleotide sequence having the sequence shown as SEQ ID No. 2.
- 30 9. A construct comprising or expressing the invention according to any one of claims 1 to 8.

10. A vector comprising or expressing the invention of any one of claims 1 to 9.
11. A plasmid comprising or expressing the invention of any one of claims 1 to 10.
- 5 12. A transgenic organism comprising or expressing the invention according to any one of claims 1 to 11.
- 10 13. A transgenic organism according to claim 12, wherein the transgenic organism is a plant.
14. A modified carbohydrate (preferably starch) prepared by a method comprising or expressing or using the invention according to any one of claims 1 to 13.
- 15 15. An enzyme substantially as described herein.

FIGURE 1

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5 ATGAGCACAGAAAAGAAAAGATGATTGCTGGT 1680
GAGTTGTATCGCTCGGCAGATGAGACGTTATCTCGCGATCGCCTGCCGCTCGTCAGCTT 1740
ATTCACCGATACAATCATTCCCTGGCGGAAGAGCACACATTACGCCAGCAAATTCCTCGCT 1800
GATCTATTCCGGTCAGGTGACAGAGGCTTATATTGAGCCAACGTTTCGCTGTGACTATGGC 1860
TATAACATTTTTCTCGGTAATAATTTTTTCGCCAACTTCGATTGCGTGATGCTTGATGTC 1920
10 TSCCCTATTCCGATCGGTGATAACTGTATGTTGGCACCAGGCGTTCATATCTACACGGCA 1980
ACACATCCCATCGACCTGTAGCACGTAATAGCGGTGCTGAACTGGGGAACCCGTCACC 2040
ATCGGTAATAACGTCTGGATTGGCGGACGCGCGGTCATTAAACCTGGTGTGACCAATTGGT 2100
GATAACGTGGTGGTAGCCTCAGGTGCAGTTGTCACAAAAGATGTCCCGGACAACGTTGTC 2160
GTGGGCGGTAATCCAGCCAGAATAATTAATAAATTGTAA

15

FIGURE 2

MSTEKEKMIAGELYRSADETLSDRLRARQLIHRYNHSLAEHTLRQQIL 50
20 ADLFGQVTEAYIEPTFRCDYGYNIFLGNNFFANFDCVMLDVCPIRIGDNC 100
MLAPGVHIYTATHPIDPVARNSGAELGKPVITIGNNVWIGGRAVINPGVTI 150
25 GDNVVVASGAVVTKDVPONVVVGGNPARIKKL 183

FIGURE 3

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	GAATTCGCCAAAGACTTGATGGATAAAGAAGGTAAAGGTCTGATTGAAGCGACGCTTGAT	60
5	GCGGTGCGGATGCGTTTACGTCCGATCCTGATGACCTCGCTGGCGTTTATCCTCGGCGTT	120
	ATGCCGCTGGTTATCAGTACTGGTGCTGGTTCGGCGCGCAGAACGCAGTAGGTACCGGT	180
	GTAATGGGCGGGATGGTGACCGCAACGGTACTGGCAATCTTCTTCGTTCCGGTATTCTTT	240
	GTGGTGGTTCGCGCGCGCTTTAGCCGCAAGAATGAAGATATCGAGCAGCCATACTGTC	300
	GATCATCATTGATACAACGTGTAATCACTAAGGCCGCGTAAGCGGCGCTTTTATGCATA	360
10	ACCTACGAACATTAAGGAGTAATTGAACCACTCAGGATCTCATACGAAAACAGTA	420
	TTAACCGGATAAAATTCATAAAAAATACTGATTGTTAGTTAATTTATATTAAGTAGCG	480
	CTAATAGATTTAATAATCCATAATCATTTAGAGGCTATTCTTAATTATTTGCGGTAATTC	540
	TTTATTCATTCTCGGTTATTACGTCATATTCAGAGCAATCCTGGTATTAGTGTACCAA	600
	TTTCATCTGGCGATAATCCTGAAATGTTATGAATAGTTCGAGCAAACTGCTTTTACCTGC	660
15	TGCGGGTTAGTGCTAGTATGAAAAAGTGAGTCCTGTCCGCTTCTTCTAATTGTAATT	720
	TTTCGTAATAATGCGATGAAAACCTGCAAAGAGTGCGCTTATAGTTAAGCTAACAAACGAG	780
	AGGGCAAGTCCAGGTCAGTAAGTTTTTTCATCCCGAAAGGTGTCCGTTAGTTCAACCGC	840
	TAAGAAGGGGACGCGTTATGGATGAATACTCAGCCAAAAGACATGATATCGCACAGCTTA	900
	AGTTTCTCTGTGAAACCTGTATCATGACTGCCCTTGCAAACCTTGAAGAAAGCAATCATG	960
20	GCTGGGTAACGACCCAACCTCGGCGATCAACCTCCAGTTGAATGAACTGATTGAGCATA	1020
	TTGCGACCTTCGCACTTAATTACAAAATTAAGTATAATGAAGACAATAAGCTCATGAGC	1080
	AGATCGACGAATATCTGGATGACACCTTTATGTTGTTCACTAGTTATGGTATTAATATGC	1140
	AGGATCTTCAGAAATGGCGGAAGTCAGGTAHCGACTATHCCGTTGTTTTGTCAATGCGA	1200
	CGAAAGAGAATCCTGCGAGTTTATCTTGTAGAATTATTACAACCATAGGTAGAAGTATG	1260
25	TCCGAAAAACCTTTAACGAAAACCGATTATTTAATGCGTTTACGTGCTGCCAGACAAAT	1320
	GACACGCTGGAGCGGTTTAWTCGAGAAAAATAATACGAATTATCAGATAATGAAGTGGC	1380
	GGTATTTTACTCAGCCGAGATCAGCGCTCGCGGAATTGACCATGAATAAACTGTACGA	1440
	CAAGATCCCTTCTCAGTATGGAATTTATTCGCTAATAAATAATTGCTTTTGGAGCTA	1500
	TAACCGGCTGTTTATTAAGAAATTTTATACTTTTTCGCCATGAAGACATACCTATGTGAT	1560
30	CTTTATCACACAGATGTAATGGGAACGTTCTCTTCACTGACTTTTTCGTTTACTGTGTTG	1620
	CCGCATTTTCAGCAACCGGAGTCAGTAATGAGCACAGAAAAGAAAAGATGATTGCTGGT	1680
	M S T E K E K M I A G	
	GAGTTGTATCGCTCGGCAGATGAGACGTTATCTCGCGATCGCCTGCGCGCTCGTCAGCTT	1740
	E L Y R S A D E T L S R D R L R A R Q L	
35	ATTCACCGATACAATCATTCCCTGCGGAAGAGCACACATTACGCCAGCAAATTCCTCGCT	1800
	I H R Y N H S L A E E H T L R Q Q I L A	
	GATCTATTCGGTCAGGTGACAGAGGCTTATATTGAGCCAACGTTTCGCTGTGACTATGGC	1860
	D L F G Q V T E A Y I E P T F R C O Y G	
	TATAACATTTTCTCGGTAATAATTTTTTCGCCAATTCGATTGCGTGATGCTTGATGTC	1920
40	Y N I F L G N N F F A N F D C V M L D V	
	TGCCCTATTCGCATCGGTGATAACTGTATGTTGGCACCAGGCGTTCATATCTACACGGCA	1980
	C P I R I G D N C M L A P G V H I Y T A	
	ACACATCCCATCGACCTGTAGCACGTAATAGCGGTGCTGAACTGGGGAAACCGTCACC	2040
	T H P I D P V A R N S G A E L G K P V T	
45	ATCGGTAATAACGTCTGGATTGGCGGACGCGCGGTCAATTAACCTGGTGTGACCATTTGGT	2100
	I G N N V W I G G R A V I N P G V T I G	

GATAACGTCGTGGTAGCCTCAGGTGCAGTTGTCACAAAAGATGTCCCGGACAACGTTGTC 2160
D N V V V A S G A V V T K D V P D N V V
5 GTGGGCGGTAATCCAGCCAGAATAATTAAAAAATTGTAATCGGTTTTTCGCAACTGTTAT 2220
V G G N P A R I I K K L
GCAAAATGTGGTAGATCTGTTACTTCCCTCTACTATTCACGTTAAAAATAGGGTGTT 2280
CCCTGGAAAGTTGCAGATACCAGGAAGGCAAACGATGACCGAAATACAACGCCTGCTGAC 2340
CGAAACGATTGAGTCTCTGAATACCCGCGAAAAACGCGACAACAAACCCGCTTTAGTAT 2400
10 CAGTTTTATCCGTAAACATCCGGGGCTGTTTATCGGTATGTACGTTGCTTTTTTTGCCAC 2460
CCTGGCGGTGATGTTGCAGTCCGAAACGCTGTCAGGCTCTGTCTGGCTACTGGTTGTATT 2520
ATTTATCCTGCTTAATGTTTTCTCTTTTTTCGATGTCTACCCACGCTACCGCTATGAAGA 2580
TATCGACGTGCTGGATTTCCGCGTTTGCTATAACGGCGAATGGTACAACACGCGCTTTGT 2640
ACCTGCCGCGCTGGTTGAAGCCATCTTGAACCTCTCCGTGTCGCGGATGTTTATAAGGAAC 2700
15 AACTGCAAAATAATGATCGTCCGTAAAGGTGAACCTGTCTTTTACGATATTTTACCCCTCS 2760
TCGCGCGGAATCAACATCTTAAGTTAGGGTTACATACCAGGCGTAAAGCTCTGCGCCTGG 2820
TCAAATGACAATGATCGTTTCCACCCATCACTTCATGAAATACCAGCTCTACCTCCTTAT 2880
CTCCAGCCAGCCTTTTTCCACAATCAGATATACTTCCCTACACTGTGTTAATAAGGATA 2940
TGCTGGTGACAACACGACATCTGGTCGGCCTTATTTCCGGGAGTACTGATTCTTTTCACTAT 3000
20 TGCTGCCTGTGCGCTTAAGCATCTGGCTGGCCCATCAGCAGGTAGAAACATCGTTTATTG 3060
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GGAAAGATGCGCTGCAG 3137

25

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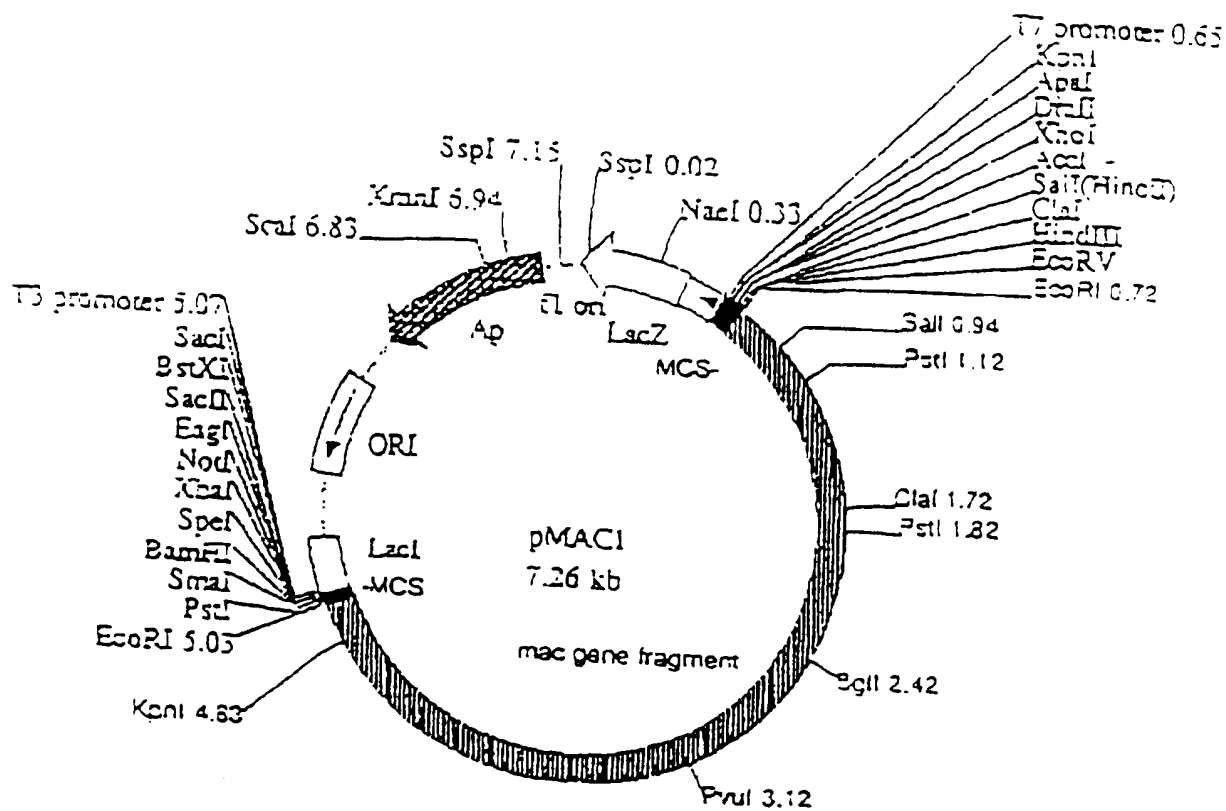


Fig 4

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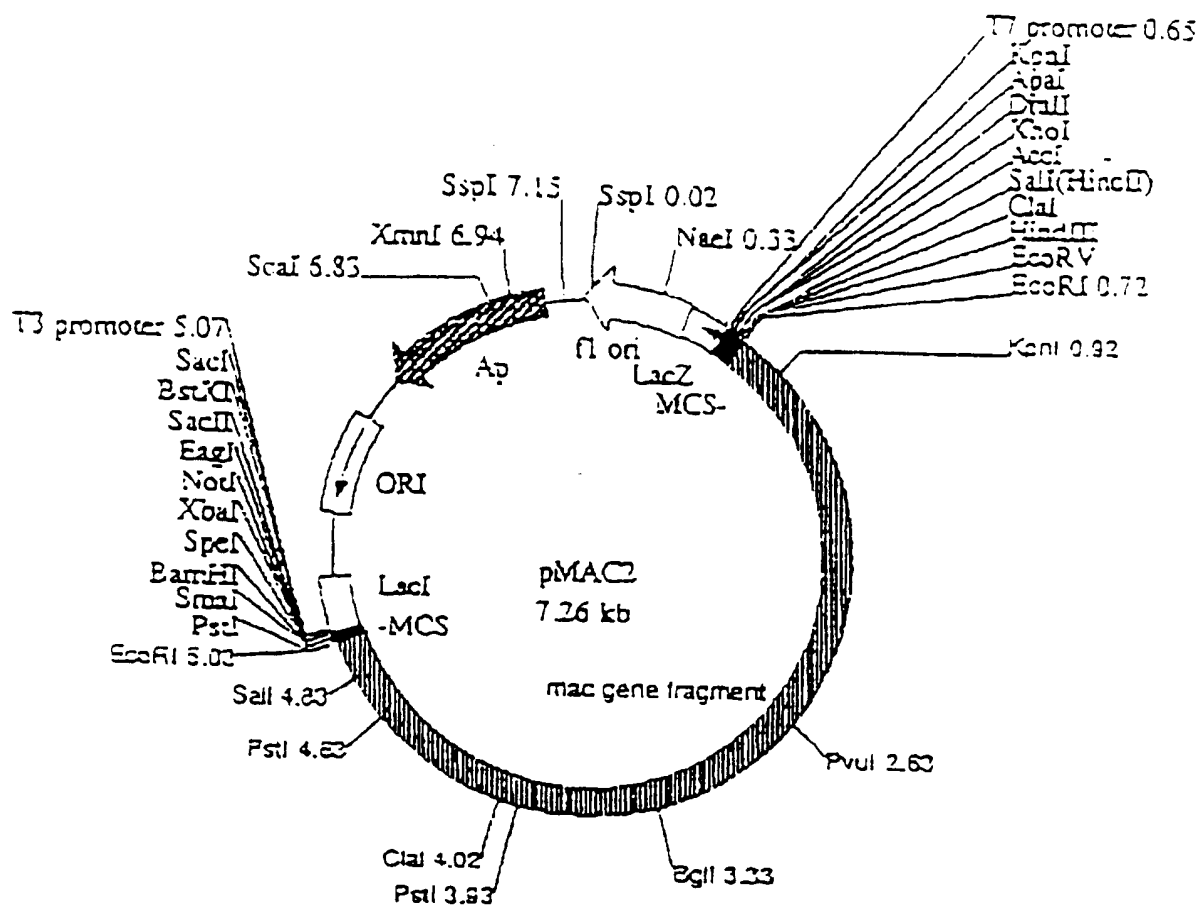


Fig 5

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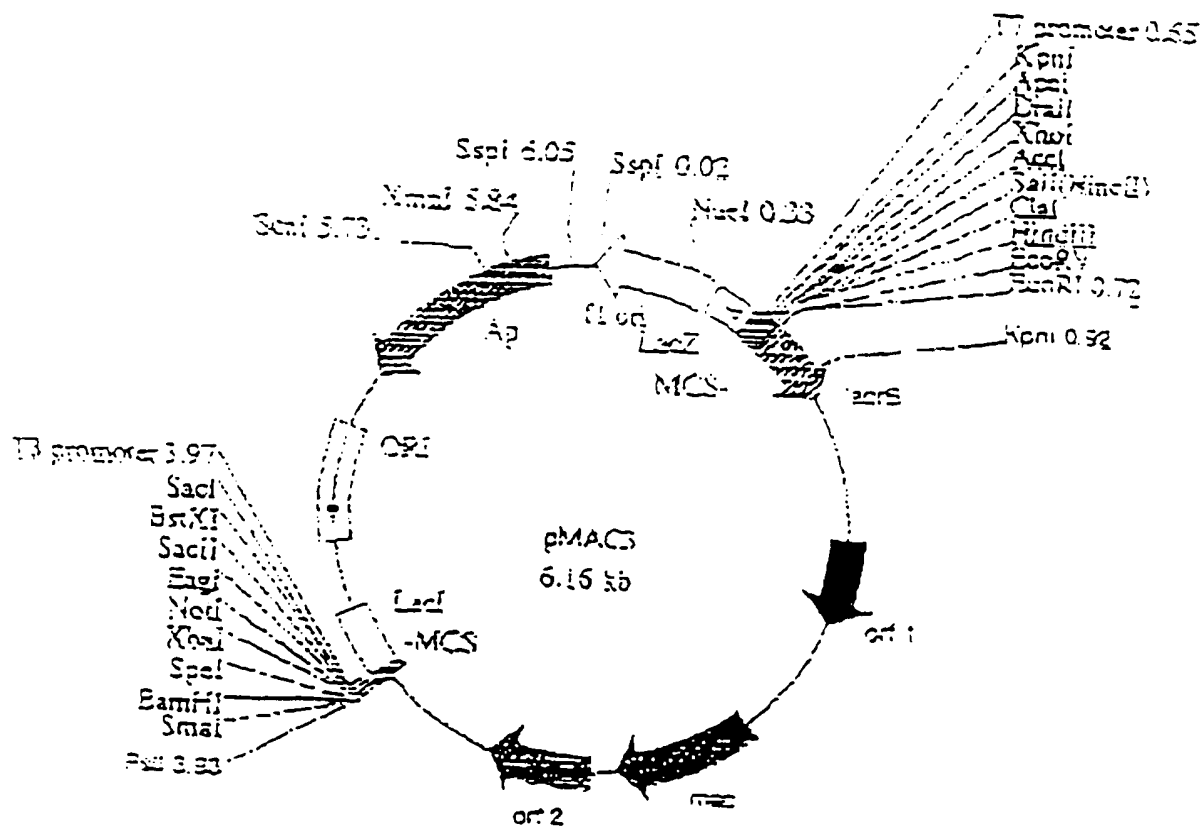


Fig 6

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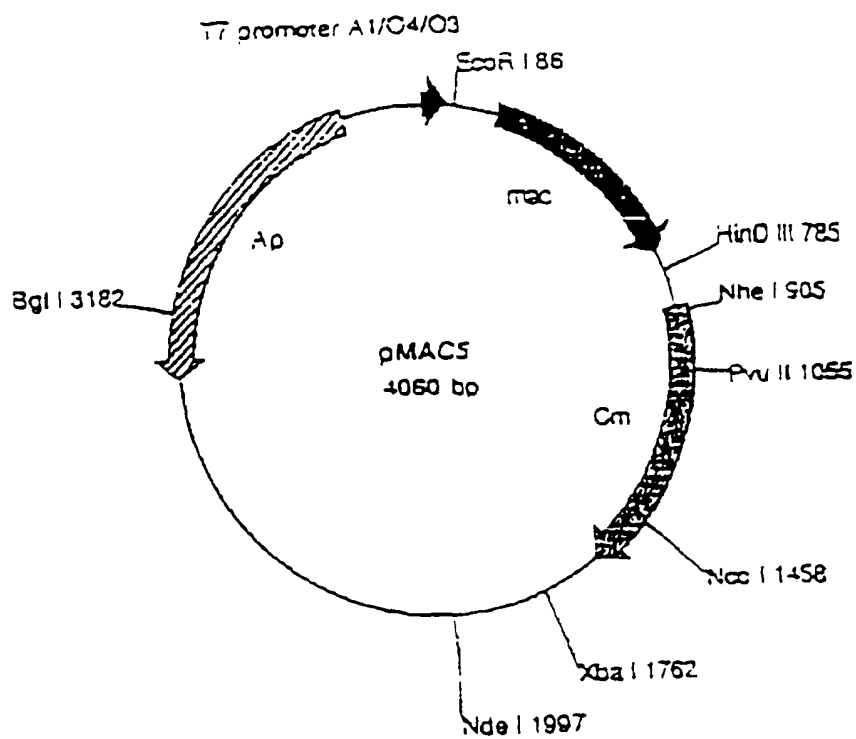


Fig 7

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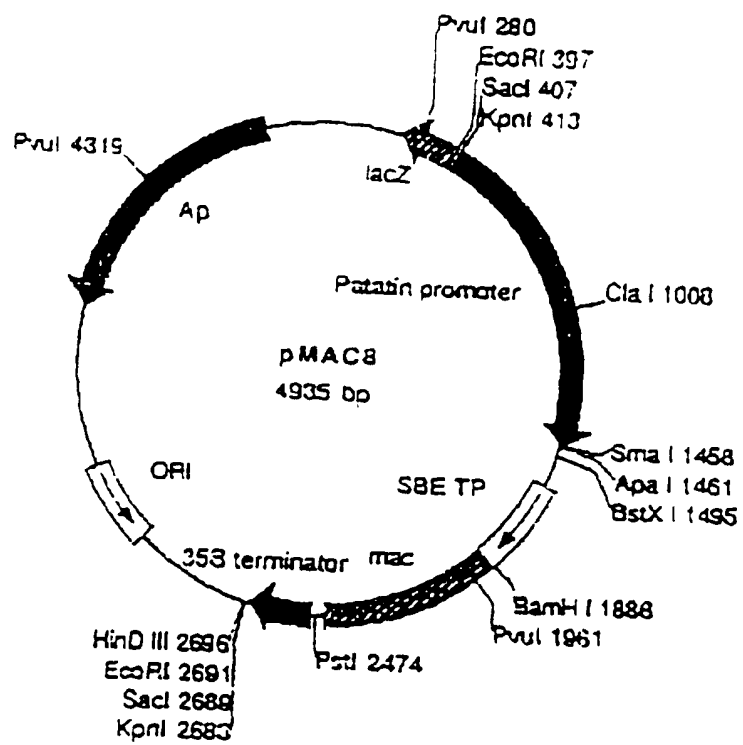


Fig 8

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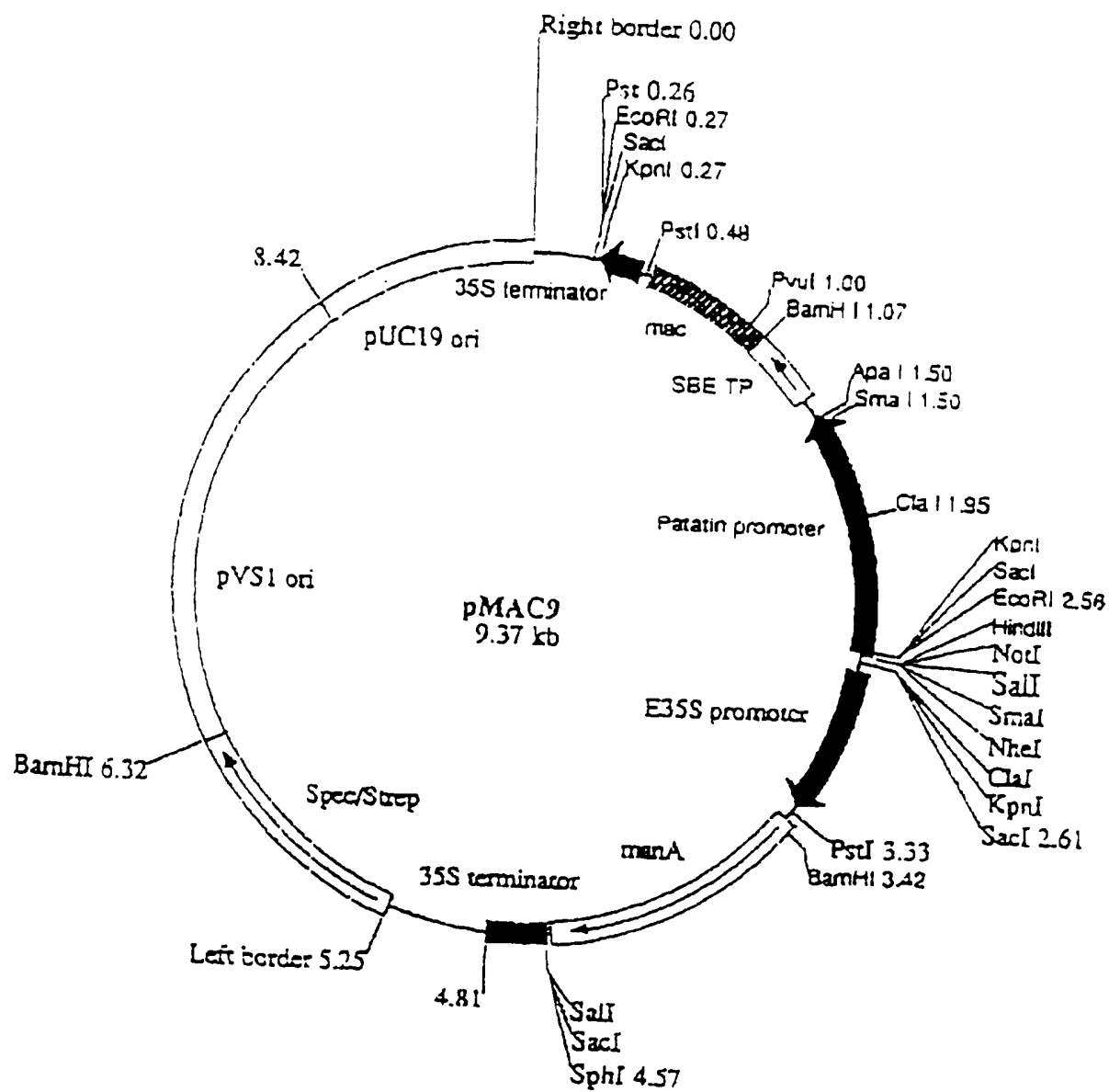


Fig 9

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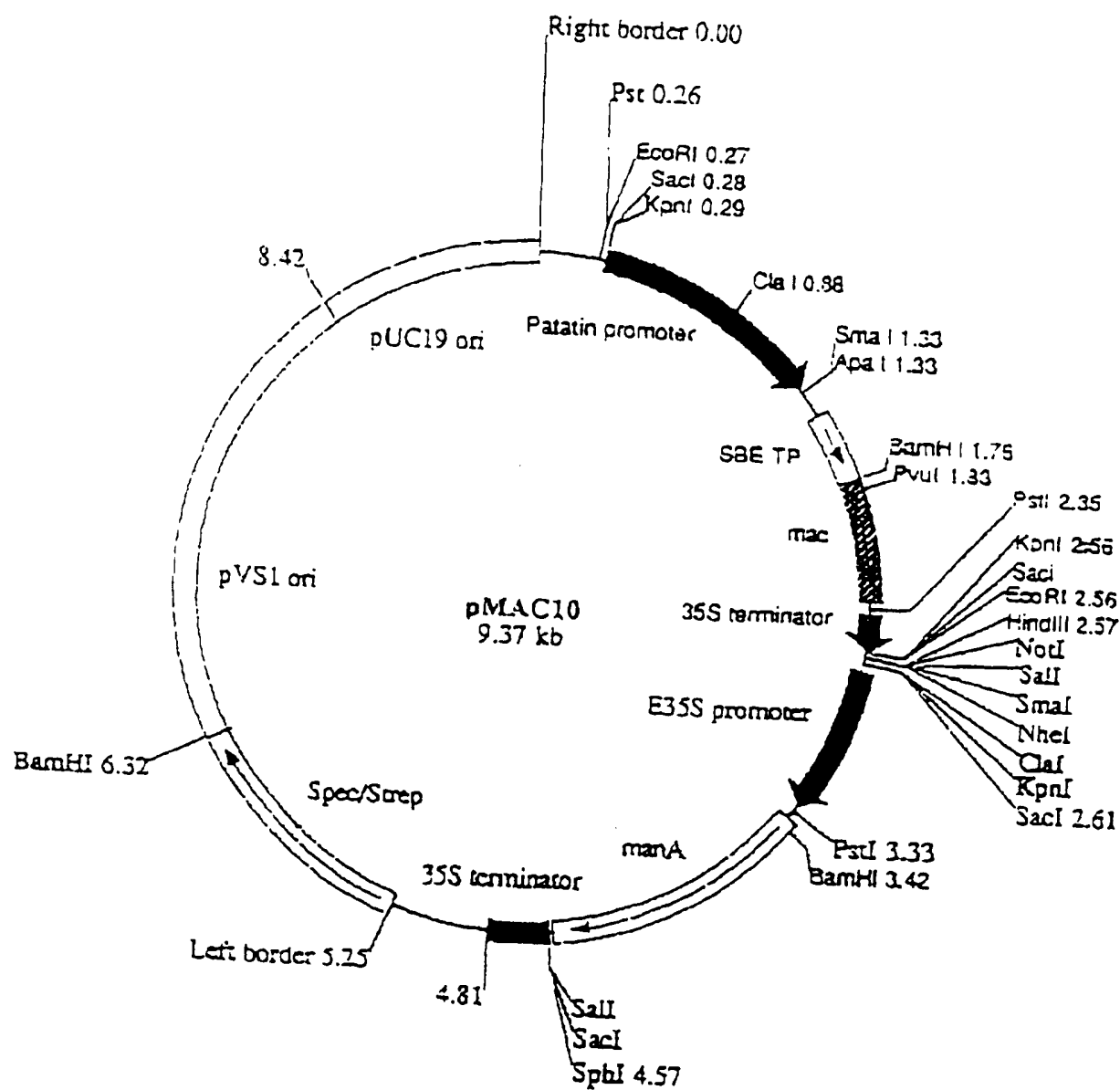


Fig 10